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(54) Title: A METHOD OF DETECTING THE PRESENCE OF AN ANALYTE IN A BIOLOGICAL SAMPLE

(57) Abstract

The present invention relates generally to a method of detecting the presence of an analyte in a biological sample. A first method for detecting blood in a biological sample involves applying asample to a first region of a test matrix, detecting globin or haemoglobin by means of an immunological test in a second region of the test matrix and detecting haem by means of a chromogen based test in a third region of the test matrix. This method is capable of differentiating between upper and lower gastrointestinal tract bleeding and is useful for the diagnosis of gastrointestinal tract diseases, in particular, lower gastrointestinal tract diseases such as colorectal cancer. A second method disclosed involve s applying a sample to a first region of a test matrix, the sample is allowed to flow to a second region where it contacts an anti-analyte immunointeractive molecule and a labelled anti-analyte immunointeractive conjugate. Any analyte-anti-analyte complex that is formed is immobilised and detected in this second region, any uncomplexed labelled anti-analyte immunointeractive conjugate is allowed to flow to a third region where it is detected.

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A METHOD OF DETECTING THE PRESENCE OF AN ANALYTE IN A BIOLOGICAL SAMPLE

FIELD OF THE INVENTION

The present invention relates generally to a method of detecting the presence of an analyte in a biological sample. More particularly, the present invention provides a method of detecting the presence of blood in a biological sample and still more particularly, the presence of high concentrations of blood. The method of the present invention also facilitates the differentiation of upper and lower gastrointestinal tract bleeding. The method of the present invention is useful, *inter alia*, for the diagnosis of gastrointestinal tract diseases and, in particular, lower gastrointestinal tract diseases such as colorectal cancer.

BACKGROUND OF THE INVENTION

Bleeding into the bowel is currently the best early indicator of bowel cancer (also known as colorectal cancer). Testing for systems of bleeding into the bowel is usually achieved by screening stools for the presence of blood. This test is often referred to as faecal occult blood testing (referred to as "FOBT").

Chemical tests are most widely used for FOBT. These tests typically require stool to be applied to paper impregnated with the chemical reagent guaiac. When developer solution is added to the paper, a blue colour develops with a positive result. Guaiac tests have the advantage of being inexpensive and easy to perform, but are less accurate (not specific for human blood) and less sensitive than desirable. Nevertheless, several international studies have shown that screening patients with these tests can save lives through the early detection of pre-cancerous and cancerous lesions. The commonly used guaiac tests detect the haem of haemoglobin, and as this is relatively resistant to breakdown in the small intestine, these tests may detect bleeding anywhere within the intestinal tract. For colorectal cancer screening this is a disadvantage as these tumours are confined to the large intestine.

Recently more sensitive and specific immunological tests (e.g. immunochromatographic tests) have been developed that have the potential to improve the accuracy of detecting blood in screening for colorectal cancer. These tests typically detect the globin protein of haemoglobin, a protein that does not survive passage through the upper gastrointestinal tract. A positive immunological test therefore indicates lower gastrointestinal bleeding. In common with all immunologically based tests, however, these tests are subject to a "prozone" or "high dose hook" effect, where at high levels of analyte, the test may be inhibited to the extent that heavy bleeding may be missed.

Accordingly, there is a need to develop improved methods of detecting blood in biological samples which methods minimise the incidence of false negative results obtained due to the effects of the prozone phenomenon. In work leading up to the present invention, the inventor has developed a method of screening biological samples for the presence of blood utilising a two part testing procedure which comprises an immunological screen for the presence of the globin component of haemoglobin performed and a non-immunological screen for the haem component of haemoglobin. Accordingly, even if the immunological detection method utilised to screen for globin produces a false negative result due to the presence of high concentrations of globin, the haem test which is not sensitive to the effects of the prozone phenomenon will nevertheless produce a positive result. The inventors have also developed an immunological screening method which overcomes the effects of the prozone phenomenon.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Accordingly, the present invention provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Accordingly, the present invention more particularly provides a method of detecting the presence of blood in a gastrointestinal sample, said method comprising the steps of :

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

According to this preferred embodiment, the present invention provides a method of detecting the presence of blood in a gastrointestinal sample, said method comprising the steps of:

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin antibody for a time and under conditions sufficient for a globin antiglobin complex to form and detecting said globin antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Accordingly, another aspect of the present invention is directed to a method of detecting lower gastrointestinal bleeding, said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of lower gastrointestinal tract bleeding.

Yet another aspect of the present invention is directed to a method of detecting upper gastrointestinal tract bleeding, said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a negative globin result is indicative of upper gastrointestinal tract bleeding.

Yet another aspect of the present invention is directed to a method of diagnosing disease conditions, the symptoms of which include bleeding, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Preferably, the present invention is directed to a method of diagnosing colorectal cancer, said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of colorectal cancer.

In a further aspect, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprising multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex

to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate;

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected.

In another further aspect, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte antibody for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte antibody conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected.

Accordingly, the present invention more particularly provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex

to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

In accordance with this more particular aspect of the present invention, in performing an analysis of the result obtained in step (ii) relative to the detection result in step (iii):

(a) a strong positive detection result at both steps (ii) and (iii) is indicative of a high analyte concentration;

(b) a weaker positive detection result at step (ii) relative to a stronger positive detection of step (iii) is indicative of a low analyte concentration;

(c) a weak positive detection result at both steps (ii) and (iii) is indicative of very high analyte concentration; and

(d) no detectable result at either steps (ii) or (iii) is indicative of extremely high analyte concentration.

Still more particularly, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said analyte, which analyte is immobilised in said third region, for a time and under conditions sufficient for an analyte-conjugate complex to form and detecting said complex;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

In still another further aspect, the present invention provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antihaemoglobin antibody for a time and under conditions for a haemoglobin-antihaemoglobin complex to form, immobilising said complex and detecting said complex by contacting said haemoglobin with an antihaemoglobin conjugate; and

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said haemoglobin, which haemoglobin is immobilised in said third region, for a time and under conditions sufficient for a haemoglobin-conjugate complex to form and detecting said complex;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the development of a blood screening method which screens for both the globin component of haemoglobin and the haem component of haemoglobin. By combining an immunological test for globin with a non-immunological test for haem, the incidence of false negative results occurring due to the prozone phenomenon are minimised. The use of a two step testing procedure directed to testing for both the haem and the globin components of haemoglobin also permits differentiation of upper gastrointestinal tract bleeding from lower gastrointestinal tract bleeding. There has also been developed an immunological based screening method which overcomes the analytically misleading test results which can be obtained when the prozone phenomenon occurs due to high analyte concentrations.

Accordingly, the present invention provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Reference to a "chromogen" should be understood as a reference to any chromogen which reacts with oxygen to produce a colour change. Chromogens suitable for use in the present

invention include, but are not limited to, guaiac, tetramethyl benzidine, ortho tolidine or functional equivalents thereof. Preferably, said chromogen is guaiac.

Accordingly, the present invention more particularly provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with guaiac or functional equivalent thereof for a time and under conditions sufficient for said guaiac to detect haem.

Reference to "biological sample" should be understood as a reference to any sample of biological material derived from an animal such, but not limited to, mucus, faeces, urine, biopsy specimens and fluid which has been introduced into the body of an animal and subsequently removed such as, for example, the saline solution extracted from the lung following lung lavage or the solution retrieved from an enema wash. The biological sample which is tested according to the method of the present invention may be tested directly or may require some form of treatment prior to testing. For example, a biopsy sample may require homogenisation prior to testing. Further, to the extent that the biological sample is not in liquid form, (for example it may be a solid, semi-solid or a dehydrated liquid sample) it may require the addition of a reagent, such as a buffer, to mobilise the sample. The mobilising reagent may be mixed with the biological sample prior to application of the sample to the test matrix or the reagent may be applied to the sample after the sample has been applied to the test matrix. The use of a mobilising reagent is required to facilitate flowing (wicking) of the

sample along the test matrix. Preferably, the biological sample is a gastrointestinal sample. By "gastrointestinal sample" is meant any sample which is derived from the gastrointestinal tract. For example, faeces, mucus (for example the mucus from a rectal mucus swab), enema wash solution or a gastrointestinal tract biopsy sample.

The term "animal" as used herein includes a human, primate, livestock animal (e.g. sheep, pig, cow, horse, donkey), laboratory test animal (e.g. mouse, rat, rabbit, guinea pig), companion animal (e.g. dog, cat), captive wild animal (e.g. fox, kangaroo, deer), aves (e.g. chicken, geese, duck, emu, ostrich), reptile or fish.

Accordingly, the present invention more particularly provides a method of detecting the presence of blood in a gastrointestinal sample said method comprising the steps of :

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Preferably said chromogen is guaiac or functional equivalent thereof.

Reference to "test matrix" is a reference to any device which is suitable for sequentially testing a biological sample for the presence of the globin component of haemoglobin, utilising a immunological test, and the haem component of haemoglobin, using a chromogen or functional equivalent thereof. In a particularly preferred embodiment, said test matrix is a

chromatographic test strip which comprises a first region for receiving a biological sample and a second region which comprises two sections. The first section of the second region is an area of immobilised antiglobin antibody coupled to colloidal gold particles which are re-suspendible by a passing liquid front while the second section of the second region is an area of immobilised antiglobin capture antibody. The third region comprises an absorbent pad impregnated with guaiac. Alternatively, the third region may comprise a strip of guaiac impregnated paper which is laminated to the second region. It should be understood that the three regions detailed in the present invention may be positioned sequentially or in some other manner, such as superimposed. For example, the first and second regions may be combined such that the sample is deliverable directly into the second region. The test matrix of the present invention may also comprise additional regions. For example, the present invention envisages the use of chromatographic strips which comprises an absorbent pad located after the third region.

Without limiting the present invention to any one theory or mode of action, according to a preferred aspect of the invention the biological sample which is applied to the first region wicks from the first region to the second region and the detection of globin and haem is then performed as a sequential two step procedure. At the second region, the globin component of any haemoglobin which is present in the sample is bound by the antiglobin antibody coupled to the colloidal gold particles. The passing biological sample front re-suspends these antibodies and both the globin-antiglobin complex and the free anti-globin antibody wick from the first section of the second region to the second section. At the second section the globlin component of any haemoglobin present in the sample becomes bound to the immobilised antiglobin capture antibody while free antiglobin coupled to colloidal gold, the non-globin components of the biological sample and any excess globin which is not bound by the anti-globin antibodies of the second region continued to wick into the third region. At the third region, the haem component of any haemoglobin which has not been captured at the second region reacts with a developer solution to cause the release of oxygen, which oxygen reacts with a chromogen such as guaiac to result in a colour change.

In the event that a biological sample comprises high concentrations of blood, and therefore high concentrations of haemoglobin, a false negative result may be obtained at the second region of the test matrix due to the prozone phenomenon. In this event, the third region of the test matrix, which detects the haem component of haemoglobin based on the non-immunological chromogen reaction, will nevertheless produce a positive result. Accordingly, the incorporation of a non-immunological chromogen test with the immunological globin test provides a safe guard against obtaining false negative results due to the effects of the prozone phenomenon where high concentrations of blood are present in the sample.

The method of the present invention requires that the haemoglobin of the red blood cells is exposed prior to commencement of the test. This may be achieved by any one of a number of methods known to those skilled in the art. For example, contacting the biological sample with a red blood cell lysis solution, prior to commencement of the test, would achieve this object. It is also within the scope of this invention to cleave the haem and the globin components of the haemoglobin either before the test begins or at some point before the biological sample wicks into the third region. In this way, it would be possible to minimize the incidence of the haem component being trapped by the antiglobin capture antibodies by virtue of its attachment to the globin component.

Reference to "immunointeractive molecule" should be understood as a reference to any molecule comprising an antigen binding portion or a derivative of said molecule. Examples of molecules contemplated by this aspect of the present invention include, but are not limited to, monoclonal and polyclonal antibodies (including synthetic antibodies), hybrid antibodies, humanised antibodies, catalytic antibodies) and T cell antigen binding molecules. Preferably, said immunointeractive molecule is an antibody.

According to this preferred embodiment, the present invention provides a method of detecting the presence of blood in a gastrointestinal sample said method comprising the steps of:

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said gastrointestinal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin antibody for a time and under conditions sufficient for a globin antiglobin complex to form and detecting said globin antiglobin complex; and

(iii) permitting flowing of said gastrointestinal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

More preferably, said biological sample is a faecal sample. Even more preferably, said chromogen is guaiac or functional equivalent thereof.

Reference to "functional equivalents" should be understood as a reference to fragments, parts, portions, mutants, homologues, mimetics from natural, synthetic or recombinant sources including fusion proteins which exhibit chromogen activity. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins.

The term "functional equivalents" as used herein should also be understood to encompass molecules exhibiting any one or more of the functional activities of a chromogen, such as, for example, products obtained following natural product screening.

Reference to a biological sample being "placed in contact" with an immunointeractive molecule or a chromogen should be understood as a reference to any method of facilitating the interaction of any one or more components of the biological sample with the immunointeractive molecule or the chromogen such that coupling, binding or other association between the one or more components of the biological sample and the immunointeractive molecule or a chemical reaction involving one or more components of the biological sample such that the chromogen colour change may occur (such as one or more components of the biological sample reacting with the developer to cause the release of oxygen which oxygen reacts with the chromogen to cause a colour change). For example the biological sample may be applied to a chromatographic strip which is impregnated with the immunointeractive molecule at the second region and the chromogen at the third region. In this instance the action of the biological sample wicking up the strip to the regions of impregnation place the sample in contact with the immunointeractive molecule or the chromogen. Alternatively, the biological sample may be applied to a chromatographic strip and the immunointeractive molecules or the chromogen may be added to the test strip at the time of testing such as simultaneously with the application of the biological sample or sequentially following the application of the biological sample. Also encompassed within the scope of "placed in contact" are combinations wherein one or more of the immunointeractive molecules, detection reagents, the chromogen or developer therefore are impregnated in the strip and others of these reagents are added to the strip at the time of testing.

"Detecting" the formation of a globin-antiglobin complex or the chemical reaction between haem and the chromogen may be by any convenient method which will be known to those skilled in the art. In the method of the invention exemplified herein, the antiglobin antibody which becomes resuspended by the wicking biological sample front is complexed with colloidal gold. As the globin-antiglobin/colloidal gold complex is trapped by the antiglobin capture antibody impregnated in the second section of the second region of the chromatography strip, the colloidal gold becomes visible as a pink band due to its increasing concentration during trapping of the complex at this point. Alternatively, the antiglobin antibody may be radio-labelled or enzymatically labelled such that upon addition of a substrate a colour change is observed if globin is present. The detection of haem by a chromogen is

preferably achieved by the addition of a developer such as peroxide which reacts with haem to produce water and oxygen. The oxygen which is liberated then reacts with the chromogen to produce a colour change. For example, when guaiac reacts with oxygen a blue colour is produced. The chromogen may be incorporated into the test matrix at the third region together with the developer or else the developer may be added as a liquid reagent at a later stage. If the developer is dried into the test matrix with the chromogen, then the paper will turn blue upon the arrival of aqueous haemoglobin. Alternatively, some other type of reporter molecule which detects the reactivity between the haem and the chromogen or functional equivalent thereof may be used.

In a preferred aspect, the present invention is used to diagnose gastrointestinal tract bleeding by analysing faecal samples for the presence of blood. Without limiting the present invention to any one theory or mode of action, the chromogen test will positively identify bleeding from any part of the gastrointestinal tract (that is, both the upper and lower regions of the tract) since it detects the haem component of haemoglobin and haem is relatively resistant to breakdown in the small intestine (the upper gastrointestinal tract). The globin component of haemoglobin however, does not survive passage through the upper gastrointestinal tract. A positive globin result in a faecal sample therefore indicates that bleeding has occurred in the lower gastrointestinal tract. Accordingly, by applying a combined two step immunological and non-immunological based test, it is possible to differentiate between upper and lower gastrointestinal tract bleeding wherein a positive haem result together with a negative globin result indicates upper gastrointestinal tract bleeding and a positive haem result together with a positive globin result indicates lower gastrointestinal tract bleeding. This is of particular importance, for example to the diagnosis of colorectal cancer, the symptoms of which include lower gastrointestinal tract bleeding.

Accordingly, another aspect of the present invention is directed to a method of detecting lower gastrointestinal bleeding said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of lower gastrointestinal tract bleeding.

Preferably said chromogen is guaiac or functional equivalent thereof.

Yet another aspect of the present invention is directed to a method of detecting upper gastrointestinal tract bleeding said method comprising the steps of:

(i) applying a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a negative globin result is indicative of upper gastrointestinal tract bleeding.

Preferably said chromogen is guaiac or functional equivalent thereof.

Yet another aspect of the present invention is directed to a method of diagnosing disease conditions, the symptoms of which include bleeding, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

Preferably, the present invention is directed to a method of diagnosing colorectal cancer said method comprising the steps of:

(i) apply a faecal sample to a first region of a test matrix which test matrix comprises first, second and third regions;

(ii) permitting flowing of said faecal sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said faecal sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of colorectal cancer.

Preferably said chromogen is guaiac or functional equivalent thereof.

The inventors have also surprisingly determined that by screening for the presence of unbound conjugate, in addition to screening for the analyte of interest, the effects of the prozone phenomenon can be overcome where an immunological screening technique is utilised. Without limiting the present invention to any one theory or mode of action, where an immunological test screen is utilised to detect the presence of an analyte, which method relies on the capture of the subject analyte by both an immobilised immunointeractive molecule and a labelled immunointeractive molecule conjugate which ultimately provides the detection means, by analysing the level of unbound detection molecule relative to the analyte test result, the test results which often arise due to prozone effects, and which are often misleading, can be interpreted correctly.

Accordingly, in another aspect, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprising multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate;

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected.

It should be understood that the terms "biological sample", "test matrix", "immunointeractive molecule", "detecting" and the phrase "placed in contact" have the same meaning as previously defined. Reference to an "anti-analyte immunointeractive molecule" should be understood as a reference to any molecule which can interact with the analyte of interest to form a complex. Preferably, said immunointeractive molecule is an antibody. In this regard, reference to an "anti-analyte immunointeractive conjugate" should be understood as reference to a molecule which can interact with the analyte of interest and which molecule either directly or indirectly facilitates the detection of the complexed, immobilised analyte described in step (ii), above. Preferably, the conjugate interact in an antigen specific manner with the analyte of interest. Reference to "detecting" has the same meaning as hereinbefore defined. The conjugate will act "indirectly", for example, if it requires an additional step to achieve visualisation of the analyte complex. For example, where the conjugate comprises an enzymatically labelled antibody, to which a substrate must be added in order to achieve a visually detectable colour change. The conjugate acts "directly" if no additional steps are required to achieve visualisation. For example, where the subject conjugate is an antibody coupled to colloidal gold, the colloidal gold will become visible as a pink band due to its increasing concentration during trapping of the conjugate.

In a preferred embodiment, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte antibody for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte antibody conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected.

Detection of the uncomplexed immunoglobin conjugate may be performed by any suitable technique. For example, the conjugate may be detected in a non-specific manner such as via an immobilised anti-immunoglobin antibody which captures the subject conjugate. Alternatively, it may be detected in a specific manner such as via a region of immobilised antigen to which the antibody conjugate is specifically directed. In a preferred embodiment, the conjugate is detected in an antigen specific manner thereby facilitating the optional inclusion of a control test to confirm that wicking of the conjugate along the test matrix actually occurs. A typical control test may therefore take the form of a region of immobilised anti-immunoglobin antibody which captures a small portion of the immunoglobin conjugate. In accordance with this embodiment, detection of the uncomplexed conjugate is achieved in an immunologically specific manner via a region of immobilised antigen to which the conjugate is specifically directed. This antigen is preferably the analyte of interest.

The steps of immobilising the analyte of interest and detecting said analyte may be performed in any suitable manner. For example, the steps may be performed sequentially or simultaneously. In a preferred aspect, the biological sample is applied to an application region of a chromatographic strip, where it is placed in contact with a colloidal gold labelled anti-analyte antibody conjugate. Following complexation of the conjugate with any analyte present in the biological sample, the sample, together with any unbound conjugate, is allowed to wick along the chromatographic strip to a second region where it contacts an immobilised anti-analyte antibody. Any analyte present in the sample will complex with the immobilised anti-analyte antibody. Where the analyte has previously complexed with the colloidal gold conjugate, it will become evident as a pink band which darkens as the labelled antibody is immobilised in the test region and its concentration increases. Any excess unbound conjugate together with the unbound biological sample components will continue to wick into a third region of the test matrix. The third region of the test matrix comprises immobilised analyte which will complex any unbound anti-analyte conjugate.

By comparing the relative intensities of the analyte test results of the second region and the conjugate test results of the third region, it is possible to determine, with greater certainty than has previously been available, whether the analyte of interest is present in the subject biological sample. Due to the fact that this technique facilitates the correct interpretation of whether or not the analyte results of region two have been subject to the occurrence of the prozone phenomenon, the results also provide a general indication of the concentration of analyte present in the sample. For example, where the analyte of interest is haemoglobin, a biological sample which does not contain any haemoglobin will produce a negative result in the second region and a strongly positive result in the third region where all the available conjugate will ultimately become complexed with the immobilised haemoglobin. Where low haemoglobin concentrations are present in a test sample, a weak positive result would be expected in the second region while a strong signal would be detected in the third region since the low level of haemoglobin initially present in the sample would have complexed only a small proportion of the total conjugate available for testing. Where high haemoglobin concentrations are present, the prozone phenomenon will result in a weak signal being detected in the second region together with a weak signal present in the third region where severely depleted conjugate concentration would become immobilised. Where extremely high haemoglobin concentrations are present it would be expected that no signal is detected in either the second or third regions due to the lack of free unbound conjugate and the nevertheless excessive concentrations of unbound haemoglobin.

In this regard, it should be understood that reference to "weak" or "strong" detection results which are obtained via steps (ii) and (iii) are characterised as being of a weak or strong nature when analysed relative to one another. These results are not necessarily analysed relative to an objective standard, although such an objective analysis is in no way excluded from the scope of the present invention. The detection result may be assessed by any suitable means. For example, where the conjugate is designed to render a colour change, the occurrence of a colour change may be assessed either by eye or via instrumental reading. Depending on the concentration of conjugate which has been immobilised this colour change may be of a faint or intense nature, corresponding to a weak or strong result, respectively. Once the detection results have been visualised, analysis of the intensity of result obtained from step (ii) relative

to the result obtained at step (iii) will be indicative of the concentration of analyte which is present in the biological sample in terms of whether the analyte is present in low, high or very high levels.

Accordingly, the present invention more particularly provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

In accordance with this more particular aspect of the present invention, in performing an analysis of the result obtained in step (ii) relative to the detection result in step (iii):

(a) a strong positive detection result at both steps (ii) and (iii) is indicative of a high analyte concentration;

(b) a weaker positive detection result at step (ii) relative to a stronger positive detection of step (iii) is indicative of a low analyte concentration;

(c) a weak positive detection result at both steps (ii) and (iii) is indicative of very high analyte concentration; and

(d) no detectable result at either steps (ii) or (iii) is indicative of extremely high analyte concentration.

It should be understood that where the detection results obtained at step (ii) and step (iii) are equivalent, assessment of whether this result is strongly positive or weakly positive may be analysed relative to an objective standard or can be assessed by the person skilled in the art in a subjective manner based on the technical knowledge and experience which such a person would possess. Where the detection results are analysed by instrumentation it may be possible to precisely quantitate the concentration of analyte present in the sample. Where the detection results are analysed by less precise means (such as by the human eye) although a precise quantitative value is not obtained, in addition to overcoming the misleading results which are caused by the prozone phenomenon, the results obtained will nevertheless broadly indicate whether the analyte is present in low, high or very high levels. This level of information can be, nevertheless, of great value. For example, where a patient presents with symptoms of bowel cancer, obtaining a broad indication of whether the patient is bleeding mildly or severely is of assistance in planning further testing and/or treatment.

Still more particularly, the present invention provides a method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate; and

(iii) permitting flowing uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said analyte, which analyte is immobilised in said third region, for a time and under conditions sufficient for an analyte-conjugate complex to form and detecting said complex;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

In a preferred embodiment, the present invention provides a method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antihaemoglobin antibody for a time and under conditions for a haemoglobin-antihaemoglobin complex to form, immobilising said complex and detecting said complex by contacting said haemoglobin with an antihaemoglobin conjugate; and

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said haemoglobin, which haemoglobin is immobilised in said third region, for a time and under conditions sufficient for a haemoglobin-conjugate complex to form, and detecting said complex;

wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

A test directed to detecting unbound conjugate can be incorporated as an additional component of any new or existing test matrix format. For example, the test format described in

accordance with this aspect of the present invention may optionally comprise a fourth test matrix region which is impregnated with a chromogen such as guiac for the purpose of additionally and simultaneously detecting haem. This is of particular relevance where it is necessary to differentiate upper from lower gastrointestinal tract bleeding as hereinbefore described.

Further features of the present invention are more fully described in the following non-limiting Examples. It is to be understood, however, that the following description is included solely for the purpose of exemplifying the present invention.

EXAMPLE 1
CHROMATOGRAPHIC TEST STRIP

Immunochromatographic tests typically use dried immunological reagents on a test strip. Liquid sample applied to the origin of the test strip flows through the various regions so that with a positive result, a coloured line develops in the upper region of the test strip. The reagents and sample then flow into an absorbent matrix at the top of the test strip. This absorbent is most commonly an absorbent paper, such as blotting paper.

It has now been found that this absorbent paper may be impregnated with guaiac, so that addition of developer solution to the absorbent at the conclusion of the immunological test can enable:

(a) Detection of high levels of blood that may have caused inhibition of the immunological test.

(b) Detection of lower gastrointestinal bleeding.

Alternatively, a strip of guaiac impregnated paper may be inserted at the upper region of the test strip, between the immunological detection zone and the absorbent.

Figure 1 depicts a test matrix suitable for use according to the method of the present invention. The origin and the first section of the second region, which is impregnated with labelled antibody (for example, antiglobinantibody coupled to colloidal gold particles), are made of the same material, which material is a conductive paper (Ahlstrom 1281). The capture antibody which is immobilised in the second section of the second region which region is made of Millipore nitrocellulose. Typically, a functional control line is also included in this region. The chromogen may be impregnated directly in the third region (the absorbent sink) or impregnated in a paper bridge between the second and third regions.

EXAMPLE 2

DETECTING HIGH CONCENTRATIONS OF BLOOD

Blood was diluted 1:100, 1:1000 and 1:10,000 in immunological test buffer. This buffer caused lysis of the red blood cells, so that the haemoglobin was liberated into solution. The diluted blood samples were added to wells of a microtitre plate. Three negative control wells were included, each containing buffer alone.

Immunological test strips (Enterix OBT) were modified so that the absorbent paper at the top of the strip was overlaid, in liquid conductive contact, with guaiac paper taken from a guaiac test (Hemoccult Sensa, SmithKline Diagnostics Inc., USA). The modified test strips were added to the microwells and yielded the following results:

	Blood Dilution			
Result	1:100	1:1000	1:10,000	Buffer
Immunological	Very weak positive (prozone)	Positive	Strong positive	Negative (x3)
Guaiac	Very strong positive	Positive	Borderline positive	Negative (x3)

EXAMPLE 3

EXPERIMENTAL DEMONSTRATION OF THE USE OF A THIRD (ANTIGEN) LINE IN AN IMMUNOCHROMATOGRAPHIC ASSAY FOR HUMAN HAEMOGLOBIN (Hb)

The purpose of the third line was to enable distinction between low signal strength due to prozone (high Hb concentration) from that due to low Hb concentration.

Preparation of test strips with a third (Hb) line

Blood was centrifuged to separate the red cells, which were then washed once with phosphate buffered saline (PBS) to remove remaining serum components. The red cells were then lysed by diluting them 1/10 with distilled water. The lysed cells were used as the source of Hb for a third line, added downstream from the Capture (anti-human Hb) and Control (anti-goat antibody) line of immunochromatographic test strips (Agen, Brisbane, Australia, Product EN12401-021 for detection of human Hb). These test strips use as the disclosing reagent a conjugate of goat anti-human Hb conjugated to colloidal gold.

The third line was dried to immobilise the Hb on the test strip.

Testing of the test strips

Whole blood was diluted

(a) 1/100 in water = High Hb concentration

(b) 1/20,000 - low Hb concentration

When these two Hb concentrations were tested with the test strips, the results (recorded as visible signal strength) were as shown:

	(a) High Hb	(b) Low Hb
Line 1 = Capture line (anti-(Hb)	Weak	Weak
Line 2 = Control line (anti-goat)	Strong	Strong
Line 3 = Hb	Weak	Strong

Conclusion: The inclusion of the third line allowed differentiation between weak detection (capture) signals caused by high and low antigen (Hb) concentrations.

EXAMPLE 4

COMBINATION OF THE ANTIGEN REGION IMMUNOLOGICAL SCREEN FOR UNCOMPLEXED CONJUGATE AND A NON-IMMUNOLOGICAL SCREEN

If the features disclosed in both Examples 1 and 2 are combined, the following detection zones appear along the test strip:

Line 1	=	capture Ab (anti-Hb)
Line 2	=	control Ab (anti-goat Ab)
Line 3	=	analyte (Hb)
Line/zone 4	=	Guaiac (or similar) for detection of haem.

These would then allow distinction to be made between the following conditions:

Condition	Line 1 (anti-Hb)	Line 2 anti-conjugate)	Line 3 (Hb)	Line 4 (Guaiac)
No blood	-	+	+	-
Blood				
1. Upper GI	-	+	+	+
2. Lower GI				
(a) lower [Hb]	+	+	+	+ (+/-)
(b) V. high [Hb]	-	+	-	+

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

CLAIMS:

1. A method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

2. The method according to claim 1 wherein said chromogen is guaiac, tetramethyl benzidine or ortho tolidine.

3. The method according to claim 2 wherein said chromogen is guaiac.

4. The method according to claim 1 to 3 wherein said test matrix is a chromatographic strip.

5. The method according to claim 4 wherein said immunointeractive molecule is an antibody.

6. The method according to any one of claims 1-5 wherein said biological sample is a gastrointestinal sample.

7. The method according to claim 6 wherein said gastrointestinal sample is a faecal sample.

8. A method of detecting lower gastrointestinal bleeding said method comprising the steps of:

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of lower gastrointestinal tract bleeding.

9. The method according to claim 8 wherein said chromogen is guaiac, tetramethyl benzidine or ortho tolidine.

10. The method according to claim 9 wherein said chromogen is guaiac.

11. The method according to claim 8 to 10 wherein said test matrix is a chromatographic strip.

12. The method according to claim 11 wherein said immunointeractive molecule is an antibody.

13. The method according to any one of claims 8-12 wherein said gastrointestinal sample is a faecal sample.

14. A method of detecting upper gastrointestinal tract bleeding said method comprising the steps of:

(i) applying a gastrointestinal sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detecting said globin-antiglobin complex; and

(iii) permitting flowing of said sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem;

wherein a positive haem result and a positive globin result is indicative of lower gastrointestinal tract bleeding.

15. The method according to claim 14 wherein said chromogen is guaiac, tetramethyl benzidine or ortho tolidine.

16. The method according to claim 15 wherein said chromogen is guaiac.

17. The method according to claim 14 to 16 wherein said test matrix is a chromatographic strip.

18. The method according to claim 17 wherein said immunointeractive molecule is an antibody.

19. The method according to any one of claims 14-18 wherein said gastrointestinal sample is a faecal sample.

20. A method of diagnosing disease conditions, the symptoms of which include bleeding, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an antiglobin immunointeractive molecule for a time and under conditions sufficient for a globin-antiglobin complex to form and detection said globin-antiglobin complex; and

(iii) permitting flowing of said biological sample to a third region of said test matrix wherein said sample is placed in contact with a chromogen or functional equivalent thereof for a time and under conditions sufficient for said chromogen to detect haem.

21. The method according to claim 20 wherein said chromogen is guaiac, tetramethyl benzidine or ortho tolidine.

22. The method according to claim 21 wherein said chromogen is guaiac.

23. The method according to claim 20 to 22 wherein said test matrix is a chromatographic strip.

24. The method according to claim 23 wherein said immunointeractive molecule is an antibody.

25. The method according to any one of claims 20-24 wherein said disease condition is colorectal cancer and said biological sample is a gastrointestinal sample.

26. The method according to claim 25 wherein said gastrointestinal sample is a faecal sample

27. A method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matric comprising multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunointeractive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate;

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is detected.

28. A method of detecting the presence of an analyte in a biological sample, said method comprising the steps of:

(i) applying a biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-analyte immunoreactive molecule for a time and under conditions sufficient for an analyte-anti-analyte complex to form, immobilising said complex and detecting said complex by contacting said analyte with an anti-analyte immunointeractive conjugate; and

(iii) permitting flowing of uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said analyte, which analyte is immobilised in said third region, for a time and under conditions for analyte-conjugate complex to form and detecting said complex.

29. The method according to claim 27 or 28 wherein the detection result obtained in step (ii) is analysed relative to the detection result obtained in step (iii).

30. The method according to claim 29 wherein:

(a) a strong positive detection result at both steps (ii) and (iii) is indicative of a high analyte concentration;

(b) a weaker positive detection result at step (ii) relative to a stronger positive detection of step (iii) is indicative of a low analyte concentration;

(c) a weak positive detection result at both steps (ii) and (iii) is indicative of very high analyte concentration; and

(d) no detectable result at either steps (ii) or (iii) is indicative of extremely high analyte concentration.

31. The method according to any one of claims 27-30 wherein said immunointeractive molecule is an antibody.

32. The method according to any one of claims 27-31 wherein said analyte is blood.

33. The method according to claim 32 wherein said biological sample is a gastrointestinal sample.

34. The method according to claim 33 wherein said gastrointestinal sample is a faecal sample.

35. A method of detecting the presence of blood in a biological sample, said method comprising the steps of:

(i) applying the biological sample to a first region of a test matrix, which test matrix comprises multiple regions;

(ii) permitting flowing of said biological sample to a second region of said test matrix wherein said sample is placed in contact with an anti-haemoglobin immunointeractive molecule for a time and under conditions sufficient for a haemoglobin-anti-haemoglobin complex to form and detecting said complex by contacting said haemoglobin with an anti-haemoglobin immunointeractive conjugate;

(ii) permitting flowing of said biological sample and said uncomplexed conjugate to a third region of said test matrix wherein said uncomplexed conjugate is placed in contact with said haemoglobin, which haemoglobin is immobilised in said third region, for a time and under conditions for a haemoglobin-conjugate complex to form and detecting said complex; and

(iv) permitting flowing of said biological sample to a fourth region of said test matrix wherein said sample is placed in contact with a chromagen or functional equivalent thereof for a time and under conditions sufficient for said chromagen to detect haem.

36. The method according to claim 35 wherein said biological sample is a gastrointestinal sample.

37. The method according to claim 36 wherein said gastrointestinal sample is a faecal sample.

38. The method according to any one of claims 35-37 wherein said immunointeractive molecule is an antibody and said chromogen is guaiac.

39. The method according to any one of claims 35-38 wherein the detection result of step (ii) is analysed relative to the detection result of step (iii).

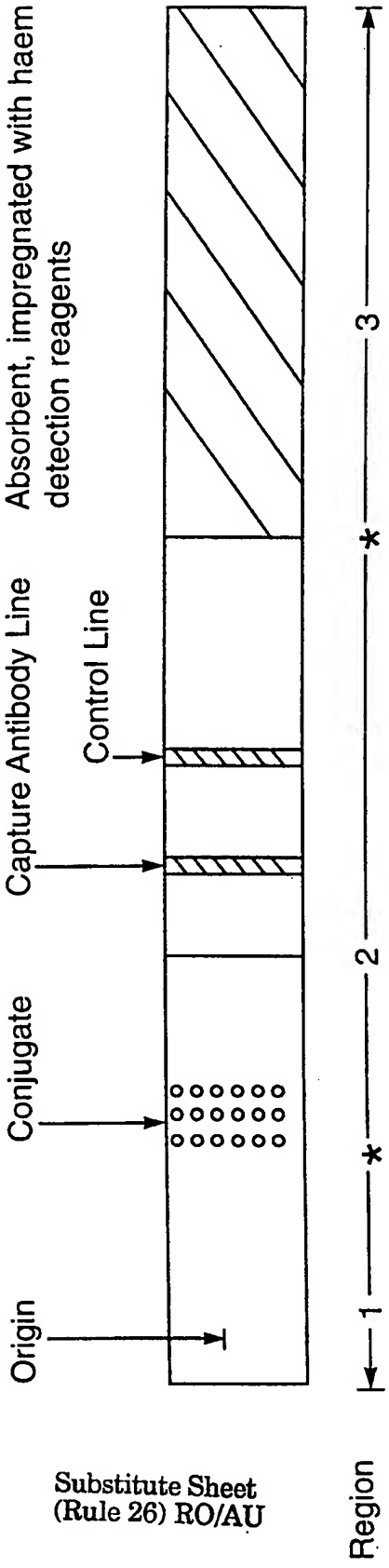
Absorbent, impregnated with haem detection reagents
Control Line
Capture Antibody Line
Conjugate
Origin
3
2
1
Region

Figure 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01014

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/72, 33/53; C12Q 1/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC Int Cl[6] G01N 33/53, 33/72; C12Q 1/28

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent (WPAT and JAPIO) and Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Am. J. Gastroenterol. (1999) February, 94(2), 344-350, *Detection of upper gastrointestinal blood with fecal occult blood tests*, Rockey D. C., Auslander A., Greenberg P. D. (see entire document, in particular page 344 and page 350 column 1)	1-3, 5-10, 1216, 18-22, 24-26
X	N. Engl. J. Med., (1996), **334**(3), 155-159, *A comparison of fecal occult-blood tests for colorectal-cancer screening*, Allison J. E. *et al.* (see entire document)	1-7,20-26
X	Derwent Abstract Accession No. 92-134663/17, Class B04 D16, HU 58917-T (Otto S) 30 March 1992. (see entire abstract)	1-7, 20-26

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2000	01 MAR 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **NORMAN BLOM** Telephone No.: (02) 6283 2238

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/01014

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	European Journal of Surgical Oncology, (1989), 15(5), 446-448, *An evaluation of Fecatwin/Feca EIA; a faecal occult blood test for detecting colonic neoplasia*, Pye G. *et al.* (see entire document)	1-7, 20-26
X, Y	AU 84188/82 (541099) B (F. Hoffmann-La Roche and Co. Aktiengesellschaft) 20 January 1983 (see entire document, in particular page 4 lines 1-3)	1-7, 20-26 8-19
Y	Clinical Chemistry, (1988), 34(9), 1763-1776, *Rapid immunochemical detection of fecal occult blood by use of a latex-agglutination test*, Vaananen P. and Tenhunen R. (see entire document, in particular pages 1763 and 1766)	8-19
X	EP 465266 A (Sangstat Medical Corporation) 8 January 1992 (see entire document, in particular page 3 lines 22-59 and the claims)	27-29, 31
X,Y	WO 98/33069 A (Smithkline Diagnostics, Inc.) 30 July 1998 (see entire document, in particular page 1 line 18, page 3 lines 4-7, page 5 lines 14-16, page 17 lines 30-33, page 25 lines 31-34, claims 6-7 etc.)	27-29, 31-34
X,Y	AU 23725/97 A (Bayer Corporation) 4 December 1997 (see entire document, in particular page 5 line 23-page 6 line 4 and claim 11)	27-29, 31
Y	WO 91/12528 A (Hygeia Sciences, Inc.) 22 August 1991 (see page 4 line 16 to page 5 line 16)	27-29, 31
X	EP 724157 A (Bayer Corporation) 31 July 1996 (see entire document, in particular columns 2 and 3, claim 3 and example II)	27-29, 31
X	WO 97/35205 A (Serex, Inc.) 25 September 1997 (see entire document, in particular page 4 lines 5-20, page 6, page 13 lines 7-28)	27-31
P, X	AU 77440/98 A (Bayer Corporation) 4 February 1999 (see entire document, in particular see claim 11, page 21 lines 6-11)	27-29, 31-32

INTERNATIONAL SEARCH REPORT

International application No.
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Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This international application was found to possess two separate inventions for the reasons given below:
(i) Claims 1-26 relate to the first invention and
(ii) Claims 27-34 relate to the second invention.
(see extra sheets for a full explanation)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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Box II

Explanation regarding finding of multiple inventions

Claims 1-26 are directed to a method of detecting the presence of blood in a biological sample which entails the following steps (i) applying a biological sample to a first region of a test matrix, (ii) permitting flow of the biological sample to a second region of the test matrix where it comes in contact with an **antiglobin immunointeractive molecule** (or antibody) which forms a globin-antiglobin complex with any globin present in the sample, and detecting this complex and (iii) permitting flow of the biological sample to a third region of the test matrix where the sample comes in contact with a **chromogen** which detects the presence of **haem**. It is to be noted that this method involves detecting the presence of two **distinct analytes** in the biological sample, each in a separate region of the test matrix. The detection of one of these two analytes involves an **immunoassay** and the detection of the second analyte is by means of a **chemical (chromogen) based** assay.

Claims 27-34 are directed to a method of detecting the presence of an analyte in a biological sample involving (i) applying the sample to a first region of a test matrix, (ii) allowing the sample to flow to a second region of the test matrix where it is contacted with an **anti-analyte immunointeractive molecule**, allowing any analyte-anti-analyte complex to form, immobilising the complex and detecting this complex by contacting it with an **anti-analyte immunointeractive conjugate** and (iii) allowing any uncomplexed **conjugate** to flow through to a third region of the test matrix where the uncomplexed conjugate is detected. This method involves the detection of a **single analyte** in the sample by means of an immunoassay in the second region of the test matrix, (the first region being the sample application region). The method also involves the detection of any uncomplexed anti-analyte immunointeractive conjugate in a further region of the test matrix. This uncomplexed conjugate which is detected in the third region is a **reagent** used for detecting the analyte in the second region and is not a second **analyte** present in the original biological sample. It is to be noted that the only methods of detecting the uncomplexed conjugate disclosed in the specification are immunoassay, and hence this method involves two immunoassays, one at each of the second and third regions.

The features common to the two groups of claims are :- (i) applying a biological sample to a first region of a test matrix, (ii) allowing the sample to flow to a second region of the test matrix where it is contacted with an anti-analyte-immunointeractive molecule, allowing any analyte present to form an analyte-anti-analyte immunointeractive complex, immobilising the complex and detecting this complex by contacting it with an anti-analyte-immunointeractive conjugate. These steps amount to nothing more than the **standard steps involved in a typical sandwich immunoassay which are very well known in the art**, examples of which are WO 96/28715, US 4960691, US 4943522 to name but a few.

The requirement of unity of invention (Rule 13.1) is fulfilled only when there is a technical relationship among those inventions involving one or more of the **same or corresponding special technical features**. The expression **special technical features** means those technical features that define a contribution which each of the claimed inventions makes over the prior art. As the method steps involving addition of a sample to a first region and allowing the sample to flow to a second region where the presence of an analyte is detected by means of an anti-analyte immunointeractive molecule amount to nothing more than a standard sandwich immunoassay one has to look to the use of a third region of the test matrix to determine any "special technical feature". The third region of the test matrix of claims 1-26 is used to detect the presence of haem (heme) using a chromogen base assay. The use of a chromogen to detect the presence of haem on a test strip is well known in the prior art and forms the basis of the currently available Hemoccult II® and HemoccultSENSA® guaiac-based tests. Likewise immunochemical tests for haemoglobin are also well known in the prior art, examples of which are HemeSelect® and HemoQuant®. The "special technical features" of claim 1-26 thus reside in the use of a **combination** of an immunoassay for globin and a chromogen based test for haem, for the detection of blood **on a single test matrix**, this being because both a chromogen based test for haem and an immunoassay for haemoglobin are both well known in the prior art, as mentioned above.

(continued)

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Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No:II

The "special technical features" of claims 27-34 relate to the detection of uncomplexed anti-analyte immunointeractive conjugate in a further separate region of a standard sandwich-type immunoassay.

These two inventions are not so linked as to form a single general inventive concept. There is no technical relationship among these two inventions involving one or more of the same or corresponding special technical features.

Further support for the above assertion of lack of unity of invention comes from the fact that a search conducted to establish the novelty and /or inventiveness of the invention defined by claims 1-26 will not establish the novelty and inventiveness of claims 27-34 and *vice versa*.

Additional evidence that the above two groups of claims define separate inventions and that one is not merely a broader form of the other can be found in the description, in particular example 4 (page 32) from which the following results have been extracted:-

For the **invention defined by claim 1-26** (test involving capture Ab (anti-Hb) and Guaiac or similar chromogen for the detection of haem)

Condition	Line 1 (anti-Hb)	Line 4 (Guaiac)
no blood lowerGI	-	-
v.high[Hb]	-	+

For the **invention defined by claims 27-34** (test involving capture Ab (anti-Hb) and anti-conjugate (Hb))

Condition	Line 1 (anti-Hb)	Line 3 (Hb)
no blood lower GI	-	+
v.high[Hb]	-	-

From these results it can be clearly seen that the two inventions measure quite different species and hence give quite different results i.e. one invention is not merely a broader form of the other invention.

Claims 35-39 are considered to possess unity of invention with each of the above two groups of claims.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/01014

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	84188/82	CA	1212024	DK	3172/82	EP	70366
		ES	513992	ES	8307899	FI	821822
		JP	58023796	NO	822458	US	4683197
HU	58917	NONE					
EP	465266	JP	6317595	US	5158869		
WO	98/33069	AU	55960/98	US	5879951		
AU	77440/98	EP	895084	JP	11083856		
WO	97/35205	AU	25531/97	CA	2249303	EP	888552
EP	724157	AU	42226/96	CA	2166913	CN	1146557
		EP	724157	JP	8240591	US	5569608
		ZA	9600357				
WO	91/12528	AU	74458/91	CA	2073504	EP	514489
		US	5141850				
AU	23725/97	CA	2198948	JP	10048212		

END OF ANNEX